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A SPIROKETALENOL ETHER PHYTOALEXIN FROM INFECTED LEAVES AND STEMS OF *COLEOSTEPHUS MYCONIS*

PETER S. MARSHALL, JEFFREY B. HARBORNE and GRAHAM S. KING*

Department of Botany, University of Reading, Whiteknights, Reading RG6 2AS, U.K.; *ICI Plant Protection Division, Jealotts Hill Research Station, Bracknell, Berkshire, U.K.

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Key Word Index—*Coleostephus myconis*; Compositae; *Botrytis cinerea*; HPLC; phytoalexin; spiroketalenol ether; myconisol; (*E*)-7-(2,4)-hexadiynyliden-1,6-dioxaspiro-[4.4]nona-2,8-dien-4-ol.

Abstract—Inoculation of stem or leaf tissue of *Coleostephus myconis* with the fungus *Botrytis cinerea* induced the biosynthesis of an antifungal spiroketalenol ether. With the aid of ^{1}H and ^{13}C NMR, IR and mass spectroscopy, the antifungal compound was identified as (*E*)-7-(2,4)-hexadiynyliden-1,6-dioxaspiro-[4.4]nona-2,8-dien-4-ol, which we name mycosinol. This is the first report of a spiroketalenol ether as a phytoalexin and the first report of a phytoalexin from a member of the tribe Anthemideae of the Compositae.

INTRODUCTION

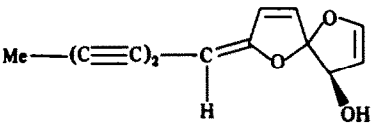
The Compositae is one of the largest families of flowering plants [1] with about 25 000 described species. However, the number of species investigated for the accumulation of phytoalexins has been small [2], probably because only a few species have any economic importance as crop plants. Phytoalexins have been reported, to date, in only three species of the Compositae, namely *Carthamus tinctorius* L. (safflower) [3], *Helianthus annuus* L. (sunflower) [4] and *Lactuca sativa* (lettuce) [5]. The phytoalexins from the safflower are the simple polyacetylenes safynol and dehydrosafynol [2] whilst the sunflower produced the coumarins ayapin and scopoletin [4] and the lettuce the sesquiterpene lactones costunolide and lettucenin A [5].

It is reported here that the leaves and stems of *Coleostephus myconis* (L.) Reich. when challenged with *Botrytis cinerea* produce two antifungal compounds, a phytoalexin mycosinol (**1**) and a photochemically derived isomer (**2**).

RESULTS AND DISCUSSION

When excised leaves or longitudinal sections of stem segments of *Coleostephus myconis* were inoculated with aqueous spore suspensions of *Botrytis cinerea*, antifungal compounds accumulated. Thin layer chromatrophy analysis of this material (silica gel, chloroform : methanol) revealed two antifungal compounds which inhibited the growth of *Cladosporium herbarum* [6] with R_f values 0.72 and 0.93. These zones of inhibition were not detected on chromatograms of uninoculated control material.

Isocratic high pressure liquid chromatography, as described in the Experimental, of extracts from the inoculated material allowed the separation of two substances which inhibited the growth of *C. herbarum* with retention times R_t 10.0 and 12.2 min. The amount of material of the first compound (**1**), was considerably greater than that of the second (**2**). **1** had a UV spectrum with λ_{max} (ε) at 242, 254 (sh), 268 (sh), 320 nm (21 000), a spectrum without fine structure typical of a diyne-ene-enol ether [7]. The mass spectrum of **1** gave a molecular ion $[M]^+$ 214 ($C_{13}H_{10}O_3$ 214) and there were prominent fragment ions at *m/z* 196 $[M-18]^+$, *m/z* 185 $(M-29)^+$, *m/z* 169 $(M-45)^+$, *m/z* 139 $[M-75]^+$, *m/z* 128 $[M-86]^+$, *m/z* 115 $[M-99]^+$, *m/z* 88 $[M-126]^+$, *m/z* 77 $[M-137]^+$, *m/z* 63, *m/z* 50. These results together with ^{1}H NMR, ^{13}C NMR and COSY experiments substantiated this structure for compound **1**, which we have for convenience named mycosinol.



1 (*E*)-isomer, mycosinol
2 (*Z*)-isomer

The second compound (R_t 12.2 min) had a similar ^{1}H NMR and ^{13}C NMR spectrum and had a UV spectrum with λ_{max} at 244, 258, 272 (sh), 308 (sh), 321, 342 (sh) nm. From these results we assign structure **2** to this compound, i.e. the (*Z*)-isomer of mycosinol.

Z-myconisol could also be derived by photochemical action on *E*-myconisol. If left in continuous daylight *E*-myconisol, a colourless solid, becomes a brown solid which was found to have the same retention time and spectral characteristics as **2**.

The tribe Anthemideae of the Compositae is a rich source of acetylenic spiroketalenol ethers [7]. Indeed, Bohlmann *et al.* [8] first isolated a C_{13}-spiroketalenol ether identical to **1** from the roots of *Chrysanthemum foeniculaceum*. This compound has subsequently been reported in the roots of other members of the Anthemideae, e.g. in *Santolina oblongifolia* [9]. However, neither **1** nor **2** have been previously reported among the constitutive acetylenic constituents of *Coleostephus myconis*.

In order to confirm that *E*-myconisol is formed *de novo* during infection, an ethereal leaf wash was prepared and subjected to the bioassay against *Cladosporium herbarum*, no zones of inhibition were produced. A concentrated ether extract of the roots was also prepared and bioassayed. The root extract was also examined by TLC to determine the presence of myconisol. *E*.-myconisol was not present in the leaf or root extracts and thus, according to the recently revised definition of the term phytoalexin [10], *E*-myconisol can be described as a phytoalexin of *Coleostephus myconis*.

Phytoalexins, by definition, should show fungitoxic activity to a range of micro-organisms. Indeed, when tested at a concentration of 100 ppm against 15 other fungal pathogens besides *Cladosporium herbarum*, it reduced spore germination and hyphal growth in all cases. It was particularly effective against *Alternaria tenuis*, which is perhaps not surprising considering that *A. chrysanthemi* is a pathogen of the garden chrysanthemum, a close relative of *Coleostephus myconis*.

The discovery of *E*-mycosinol in *Coleostephus myconis* is the first report of a spiroketalenol ether as a phytoalexin and the first identification of this type of antifungal agent in the tribe Anthemideae. Present indications are that the type of phytoalexin produced in the Compositae may be characteristic at the tribal level [3–5] and further taxa are being investigated to test this hypothesis.

EXPERIMENTAL

Plant material and the fungal cultures. Seed of *Coleostephus myconis* was obtained from the Hortus Botanicus Hauniensis, Denmark. The plants were greenhouse grown in John Innes No. 2 compost and were authenticated after flowering by reference to our herbarium collection of Anthemideae specimens. *Botrytis cinerea* was maintained on 5% malt in 2% agar and inoculum was taken from 2–3 week cultures grown at 20° on the above medium. *Cladosporium herbarum* was grown on V8 agar at 25%.

Preparation of spore suspensions. Conidia of *B. cinerea* were prepared by adding 5 ml aliquots of distilled water to the cultures and rubbing the culture with a sterile spatula. The spore suspension was then filtered through two layers of muslin to remove mycelial fragments and adjusted to the required concentration. The spore suspension of *C. herbarum* was prepared by the method of ref. [6].

Induction and isolation of myconisol. The spore suspension of *B. cinerea*, consisting of 10^6 conidia per ml in deionised H_2O, was added to plastic boxes (80 ml/box) containing blotters in the bottom. Excised leaves or longitudinally cut stem sections (15 mm in length) were placed on the blotters. Distilled H_2O was added to the control boxes. On the occasions when the quantity of myconisol needed to be increased it was found that it could be enhanced by applying droplets of 0.05 M $CuSO_4$ to the adaxial leaf surface. The boxes were incubated in continuous diffuse lighting at room temp. for 72 hr. After incubation the diffusate from leaf inoculations, now contained within the spore suspension, was collected and partitioned ×3 with equal vols of dichloromethane. The organic fraction was collected and concentrated *in vacuo* prior to chromatography.

Chromatography. TLC (silica gel G25-UV 254, 0.25 mm developed in $CHCl_3$–MeOH 96:4) produced two compounds visualised under short and long UV, 0.72 and 0.93. Myconisol could also be detected by spraying with a solution of 1% $KMnO_4$ in 2% Na_2CO_3 (aq.) [11] to give a yellow spot on a violet background.

HPLC (Hichrom spherisorb S5W column, Waters UV absorption detector at 254 nm, mobile phase 30% $CHCl_3$ in hexane, 1 ml/min) furnished myconisol, retention time 10.0 and the *Z*-stereoisomer, 12.2 min. The levels of both compounds were determined directly from peak areas using a Spectra Physics 4270 Integrator.

Bioassay technique. A similar method to that of ref. [6] was employed. Developed silica gel plates, were sprayed with a suspension of conidia of *Cladosporium herbarum*. The plates were then incubated in sealed damp boxes at 25° for 5–7 days in darkness, to locate antifungal compounds.

Preparation of an ethereal leaf wash. This involved plunging 35.75 g of leaf material into 250 ml Et_2O for 1 min. The ethereal solution was decanted off and coned *in vacuo* prior to chromatography. The developed TLC plate was then sprayed with the conidial suspension of *C. herbarum* as decribed earlier.

Preparation of the root extract. 200 g of root tissue of *C. myconis* was removed and washed clean in distilled H_2O. The fresh root tissue was chopped into small pieces and covered with Et_2O and stored at room temp. for 72 hr. The Et_2O was decanted off and dried with anhydrous $MgSO_4$ prior to concentration *in vacuo*. The concentrated extract was subjected to TLC and isocratic HPLC but no myconisol could be detected.

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REFERENCES

1. Heywood, V. H. (1978) *Flowering Plants of the World* p. 263. Oxford University Press, Oxford.
2. Coxon, D. T. (1982) in *Phytoalexins* (Bailey, J. A. and Mansfield, J. W. eds). Blackie, Glasgow.
3. Allen, E. H. and Thomas, C. A. (1971) *Phytochemistry* **10**, 1579.
4. Tal, B. and Robeson, D. J. (1986) *Phytochemistry*, **25**, 77.
5. Takasugi, M., Okinaka, S., Katsui, N., Masamune, T., Shirata, A. and Ohuchi, M. (1985) *J. Chem. Soc. Chem. Commun.* **10**, 621.
6. Homans, A. L. and Fuchs, A. (1970) *J. Chromatog.* **51**, 327.
7. Bohlmann, F., Burkhardt, T. and Zdero, C. (1973) *Naturally Occurring Acetylenes*. Academic Press, London.
8. Bohlmann, F., Arndt, H., Bornowski, H., Kleine, K. M. and Herbst, Pn (1964) *Chem. Ber.* **97**, 1179.
9. de Pascual Teresa, J., Bellider, I. S., Gonzalez, M. S. and Vicente, S. (1986) *Phytochemistry* **25**, 185.
10. Paxton, J. (1980) *Plant Disease* **64**, 734.
11. Garrod, B., Lewis, B. G. and Coxon, D. T. (1978) *Physiological Plant Pathology* **13**, 241.